Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di pusat pengajian.

LAPORAN AKHIR PROJEK PEMYELIDIKAN

R & D JANGKA PENDEK

A. MAKLUMAT AM

Tajuk Projek :	**A Study on the Protective Effects of Calcium Channel Blockers Against Anoxic Brain Damage**
Tajuk Program :	**IRPA Short Term Grant**
Tarikh Mula :	1st February, 1997
Nama Penyelidik Utama : *(berserta No. K/P)*	Dr. Sunil Gurtu (S 728210)
Nama Penyelidik Lain :	Prof. Madya S.B. Acharya Prof. Madya H. A. Nadiger Dr. Mohd. Shakil Siddiqui

B. PENCAPAIAN PROJEK :

(Sila tandakan / pada kotak yang bersesuaian dan terangkan secara ringkas di dalam ruang di bawah ini. Sekiranya perlu, sila gunakan kertas yang berasingan)

Penemuan asli / peningkatan pengetahuan

The results of the present study show that

1. Out of the three calcium channel blockers tested, administration of verapamil and flunarizine produced a significant decrease in the basal levels of MDA in the brain .
2. Verapamil and flunarizine also afforded protection against the anoxia-reoxygenation induced increase in MDA levels in brain
3. In both the above verapamil appeared to be more potent as compared to flunarizine.
4. Diltiazem does not appear to share the neuroprotective which is evident in verapamil and flunarizine.

These results provide a better understanding of the neuroprotective effects of calcium channel blockers.

Rekaan atau perkembangan produk baru,
(Sila beri penjelasan / makluman agar mudah dikomputerkan)

i) .....Not applicable.....

.....

ii) .....

.....

iii) .....

.....

Mengembangkan proses atau teknik baru,
(Sila beri penjelasan / makluman agar mudah dikomputerkan)

i) .....

.....

ii) .....

.....

iii) .....

.....

Memperbaiki /meningkatkan produk/ proses / teknik yang sedia ada :
(Sila beri penjelasan / makluman agar mudah dikomputerkan)

i) .....

.....

ii) .....

.....

iii) .....

.....

C. PEMINDAHAN TEKNOLOGI

Berjaya memindahkan teknologi.

Nama Klien : *(Nyatakan nama penerima pemindahan teknologi ini dan sama ada daripada pihak swasta ataupun sektor awam)*	i)	.....
	ii)	.....
	iii)	.....

Berpotensi untuk pemindahan teknologi.
(Nyatakan jenis klien yang mungkin berminat)

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D. KOMERSIALISASI

Berjaya dikomersialkan.

Nama Klien: i) .....

ii) .....

iii) .....

Berpotensi untuk dikomersilkan.
(Nyatakan jenis klien yang mungkin berminat)

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E. PERKHIDMATAN PERUNDINAGN BERBANGKIT DARIPADA PROJEK
(Klien dan jenis perundingan)

1) .....

2) .....

3) .....

4) .....

F. PATEN /SIJIL INOVASI UTILITI
(Sekiranya nombor dan tarikh pendaftaran paten. Sekiranya paten / sijil inovasi utiliti telah dipohon tetapi masih belum didaftarkan, sila berikan nombor dan tarikh fail paten).

1) .....

2) .....

3) .....

G. PENERBITAN HASIL DARIPADA PROJEK

(i) LAPORAN/KERTAS PERSIDANGAN ATAU SEMINAR

1) A paper is under preparation for presentation at the forthcoming FIFTH IBRO WORLD CONGRESS OF NEUROSCIENCE

2) .....

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3) .....

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4) .....

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5) .....

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(II) **PENERBITAN SAINTIFIK**

1) Manuscript entitled " Neuroprotective effects of calcium channel blockers against anoxia-reoxygenation induced brain injury in rats", is under preparation for submission to **NEUROREPORT.**

2) .....

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6) .....

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7) .....

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H. **HUBUNGAN DENGAN PENYELIDIK LAIN**
(Sama ada dengan institusi tempatan ataupun di luar negara)

1) .....

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2) .....

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3) .....

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4) .....

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I. SUMBANGAN KEWANGAN DARI PIHAK LUAR
(Nyatakan nama ajensi dan nilai atau peralatan yang telah diberi)

i) .....

ii) .....

iii) .....

J PELAJAR IJAZAH LANJUTAN
(Nyatakan jumlah yang telah dilatih di dalam bidang berkaitan dan sama ada di peringkat sarjana atau Ph.D)

	Nama Pelajar
Sarjana	.....
	.....
	.....
Ph. D	.....
	.....
	.....

K. MAKLUMAT LAIN YANG BERKAITAN

The results of the present study indicate that calcium channel blockers reduce anoxia-reoxygenation induced neuronal damage and give a comparative evaluation of three calcium channel blockers belonging to three different classes. However, in the present study we only pretreated the animals before anoxic challange. It would be interesting and perhaps more relevant clinically to see the effect of these agents when they are administered following the anoxic challange. Similarly. for the histological studies, we were unable to detect any changes in neuronal morphology using light microscopy. It would be worthwhile to see the changes in ultrastructure (using electron microscopy) in the acute phase following anoxia-reperfusion, and the effects os calcium channel blockers thereupon, if any. This study thus lays a foundation for further, more detailed exploration.

04/01/99
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Tarikh

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Tandatangan

T/TANGAN PENGERUSI
J/K PENYELIDIKAN
PUSAT PENGAJIAN

fn:r&d/borang permohonan/as

A Study on the Protective Effects of Calcium Channel Blockers Against Anoxic Brain Damage

Introduction

It is now widely accepted that a rapid neuronal influx of Ca^{2+} following cerebral ischaemia/ hypoxia is intimately connected with the cascade of events — including activation of enzymes which give rise to the production of reactive oxygen species and nitric oxide, thereby leading to free-radical induced damage —which reseult in neuronal injury and death. It is also known that a burst of free radical generation occurs during re-oxygenation of the tissues following a period of ischaemia/hypoxia as also a prolonged purturbation of the membrane functions continues after the perfusion/oxygenation has been restored, which leads to continued accumulation of calcium intracellularly , resulting ultimately in delayed damage and death of the neurone.

Estimation of lipid peroxidation products provides a reliable estimate of free radical induced damage to tissues, and has been widely used for this purpose.

Objectives

The present study was undertaken with an aim to investigate whether :

i)attempts to block the voltage dependent calcium channels with organic clacium channel blockers reduce the extent of free radical induced neuronal damage in the short term, following experimental global hypoxia in rats, as expressed by the presence of thiobarbituric acid reactive substances (TBARS) in the brain tissue.

ii)the expression of increased TBARS is also paralleled by any evidence of acute changes in neuronal morphology.

Methods

Adult rats of Wistar strain weighing betwee 150 1nd 200 gm were used for the present study. Each experimental group comprised of an equal number of rats from either sex.Prior to experiments they were grouped in seaparte cages and fed standard rat pellet chow and water *ad libitum*. They were maintained under natural laght-dark cycle. All experimental procedures were done between 0930 and 1200 and the biochemical estimations were completed within 4 hours of the sample collection.

The following experimental groups were designed:

Group 1. Control group (CON)

Group 2. Untreated rats subjected to anoxia (ANOX)

Group 3. Untreated rats subjected to anoxia followed by re-oxygenation (REOX)

Groups 4 - 6. Treated with either of the three calcium channel blockers (CCB) , verapamil (VerCON), diltiazem (DilCON) or flunarizine (FluCON)

Groups 7 - 9. Subjected to anoxia following pretreatment with verapamil (VerANOX), diltiazem (DilANOX) or flunarizine (FluANOX).

Groups 10 - 12. Subjected to anoxia and re-oxygenation following pretreatment with the three CCBs (VerREOX, DilREOX and FluREOX).

Induction of anoxia and re-oxygenation:

For induction of anoxia the rats were placed individually in one litre jars which were rendered airtight by putting a plasticine ring around their edges and then firmly placing a glass sheet over them to block air entry. The animals were allowed to breath in this closed space and were continuously observed for appearance of convulsions. In preliminary experiments it was determined that under these conditions the rats would develop convulsions after 30 minutes and would die shortly that if not allowed fresh air aftyer this stage. Since convulsions can by themselves cause widespread and unpredictable alterations in the brain biochemistry it was decided to subject the rats to anoxia for a period of 30 minutes.

At the end of this period the animals were either sacrificed (anoxia groups) or replaced in their cages to allow them normal breathing environment for one hour (re-oxygenation group) prior to sacrifice.

Decapitation and preparation of brain tissue:
The rats were sacrificed by decapitation with a guillotine, just below the first cervical vertebra and The brain was quickly dissected out and put in ice cold normal saline (10 ml). For homogenization, the brain was taken out , dried with a blotting paper, weighrd and then homogenised in ice-cold trichloroacetic acid (TCA) to make a 10% homogenate.

Histological studies
For histology, fixation of the brain was done in 10% buffered formalin solution. After two weeks, coronal sections, passing through the hippocampus were cut. The formalin fixed sections were dehydrated in ascending grades of alcohol, cleared and paraffin embedded. The paraffin embeded blocks were sectioned at 4 micron and 10 micron thicknes with a rotatory microtome. Thick sections were stained with Nissl stain using cresyl violet. Myelin staining was done with luxol fast blue . Thin sections were stained with haematoxylin and eosin stains. The sections were examined under Olympus microscope.

Estimation of thobarbituric acid reactive substances (TBARs) in the brain homogenates :
One ml of the brain homogenate (10%) , 1 ml of 40% TCA and 2 ml of 0.67 % thiobarbituric acid solution were mixed , vortexed and incubated for 10 minutes in a boiling water (100 Celcius) bath. The reaction mixture was cooled, centrifuged and the colour intensity was measured with a spectrophotometer at a wavelength of 536 nm. The concentration of MDA in terms of TBARs was calculated using the extinction coefficient of 1.56×10^{-5} M^{-1}.

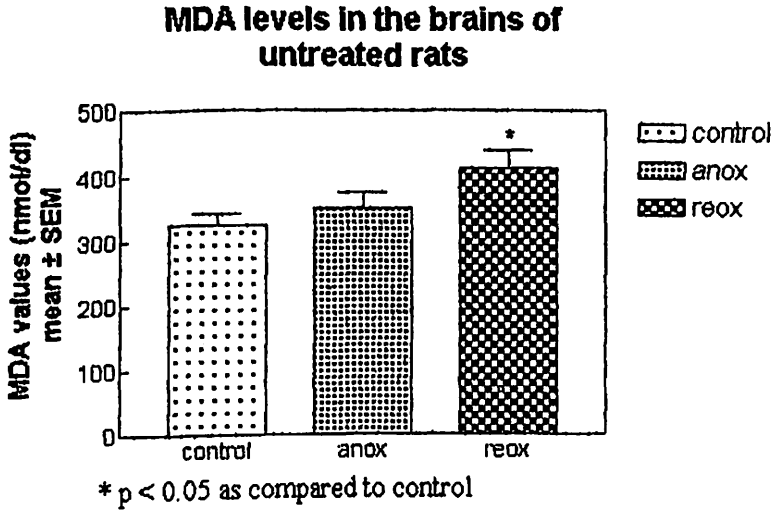
Drug administration:
All drugs were dissolved in distilled water and administered intraperitoneally in a volume not exceeding 0.2 ml. The control group was administered 0.2 ml of normal ssaline. In groups 4 - 6 drugs were administered 60 min prior to decapitation while in groups 7 - 12 they were administered 30 minutes prior to the anoxic challange. All the three drugs were administered in a dose of 5 mg per kg body weight.

Treatment of data:
The means and standard errors of the means (SEM) of the values of estimated MDA concentrations (nMol dL^{-1}) for each group were computed and subjected to Student's 't' test for determining the level of significance of the differences between the means of different groups at an error level of 5%.

Results and Conclusions

I. Biochemical Studies:
Data from a total of 120 rats were obtained and are reported below. Among the untreated groups the control, anoxia and re-oxygenation groups consisted of 24, 14 and 10 animals respectively while all the treated groups consisted of 8 animals each. The values for MDA levels are reported as mean ± s.e.m, in $ng.dL^{-1}$.

Effect of anoxia and reoxygenation on brain MDA levels in untreated rats (fig 1)



* $p < 0.05$ as compared to control

Fig 1

The mean MDA levels in the brains of rats which were allowed re-oxygenation following anoxia, were significantly higher as compared to that in the control group. However anoxia alone did not result in a

statistically significant alteration of the brain MDA levels. This observation is in agreement with the reports which describe a burst of free radical generation during the epriod immediately following reperfusion of the hypoxic tissue.

Effect of pretreatment with CCBs on the basal brain MDA content (Fig 2)

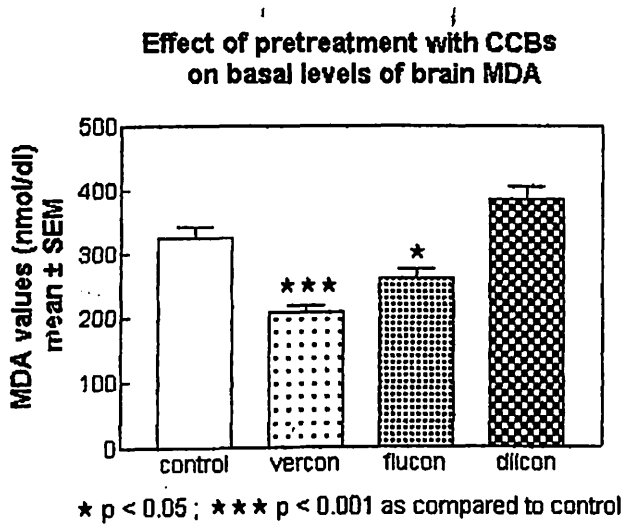


Fig 2

Pretreatment of rats with verapamil or flunarizine (5 mg/kg i.p.) 60 minute prior to decapitation elicited a significant decrease in the basal levels of brain MDA content. Dose for dose, verapamil appeared more potent as compared to flunarizine (mean MDA levels 207.4 ±10.03 and 259.9 ± 16.17 ng/dL respectively, $p< 0.01$). In contrast, pretreatment with diltiazem (5 mg/kg, i.p.) did not produce any lowering of the basal brain MDA levels. Actually there appeared a tendency towards elevation of basal MDA levels , although this was not statistically significant.

Effect of pretreatment with CCBs on the reoxygenation induced increase in brain MDA levels.

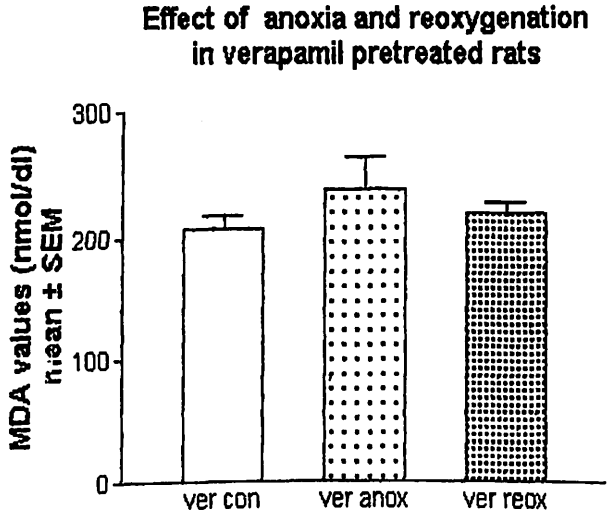


Fig 3

In the verapamil pretreated group (Fig 3), anoxia followed by re-oxygenation did not produce any significant alteration in the brain MDA content, indicating thereby that not only did verapamil cause a decraese in the basal MDA content but it also prevented the anoxia-reoxygenation induced increase in free radical damage.

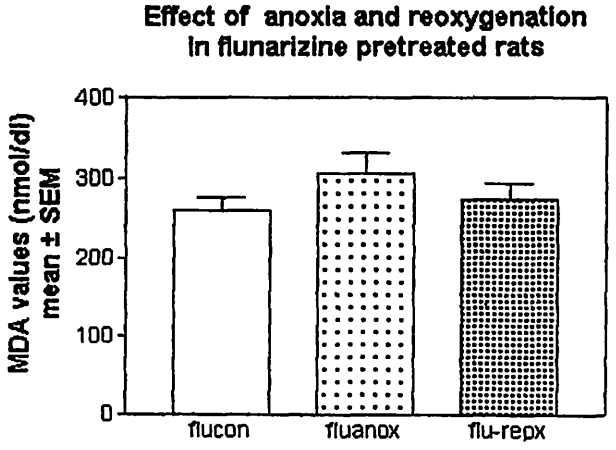
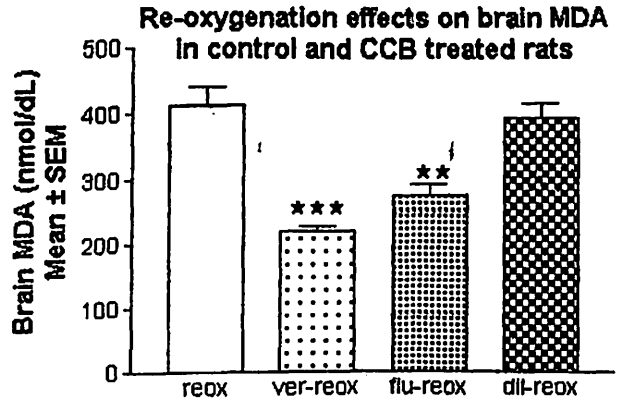


Fig 4

Although flunarizine pretreatment (Fig 4) produced relatively less decraese in basal MDA levels as compared to verapamil, yet like verapamil, it also inhibited the anoxia-re-oxygenation induced increase in brain MDA .

The effects of diltiazem require a little more detailed analysis. In this group also, the MDA values following reoxygenation when compared with those in the diltiazem-treated-unchallanged rats were not found to be statistically different, implying thereby that diltiazem alaso affords protection against reoxygenation induced peroxidative damage. However, the basal MDA values following diltiazem pretreatment (dilCON group) were higher than the control basal values, though not attaining statistically significant proportion with this sample size. Reoxygenation following diltiazem pretreatment also exhibits a trend towards increase in the mean MDA values however when compared against an already elevated basal value (dilCON) it is not statistically significant. When we compare the post reoxygenation MDA values in all the four groups (REOX, VerREOX, FluREOX and DilREOX) , the verapamil and flunarizine groups exhibit markedly lower MDA levels as compared to the control group while the MDA values in the diltiazem treated group are statistically no different from the untreated-reoxygenation group (Fig 5).



*** $p < 0.001$, ** $p < 0.01$ as compared to the reox (untreated reoxygenated) group

Fig 5

Thus, while verapamil and flunarizine pretreatments exhibit a lowering of the basal MDA levels as well as a protective action against anoxia-regeneration induced oxidative brain damage expressed as TBARs, diltiazem does not appear to share

II. Histology studies:

Light microscopic examination did not reveal any changes in the neurones subjected to anoxia or anoxia-reoxygenation. There was no neuronal loss or any degenerative changes such as hyoerchromatic nucleus, chromatolysis and shrinkage of perikaryon, microvacuolation in the mantle layer of the CA1, CA2, CA3 and CA4 regions of the hippocampus which was examined as this region is highly sensitive to anoxic stress. In the control and experimental groups, there was no morphological difference in the glial cells or in the neuropil.

Neurons in the hippocamus which are sensitive to anoxia, and exhibit early changes induced by anoxia, do so within 24 to 72 hours following anoxia (Greenfield, 1992; Izumiyama, 1988; Sutherland, 1988). In our study the animals were sacrificed within 60 - 90 min of the anoxic challange, it is possibly too early for morphological changes to become evident under light microscopy. However, changes at the ultrastructural or histochemical levels cannot be ruled out and need further conifrmation.